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The proposed studies aim to understand the biochemical mechanism of conversion of normal mammary epithelial cells (MECs) into tumorous cells. We have identified a novel serine protease-like gene NES1 that is specifically expressed in MECs but not in fibroblast cells. NES1 was isolated by virtue of its markedly reduced expression in a radiation-transformed MEC compared to its normal parent cell. NES1 showed reduced or no expression in a panel of mammary tumor cell lines. Furthermore, NES1 mRNA expression is cell cycle-regulated. NES1 is unique in that it is a protease that is down-regulated during tumorigenesis. Together, these features suggest that NES1 plays a role in a pathway that negatively regulates MEC growth. It is likely that NES1 either inactivates a positive regulator of cell proliferation or it generates an active form of a negative growth regulator through selective cleavage of an inactive form. This grant proposal represents a comprehensive structural and functional characterization of NES1 to address this hypothesis. In the long run, identification of specific targets of oncogenic processes and delineation of their precise function should allow rational design of therapeutic agents, and offer reagents for diagnosis or monitoring of treatment of breast cancer.

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FOREWORD

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Yes In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Vimla Band 9/12/97

PI - Signature Date

TABLE OF CONTENTS:

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths of women in North America and Europe. About 180,000 new cases of breast cancer are diagnosed every year in the United States alone, with one in eight women estimated to develop breast cancer during her life time. In nearly all cases the etiology of these cancers is unknown. Similar to other human cancers, the vast majority of breast cancers represents a malignant transformation of epithelial cells. Malignant transformation represents a complex multi-step process in which genetic changes and environmental factors, such as radiation, viruses and dietary factors, are thought to deregulate the cellular processes that control cell proliferation and differentiation (1). Therefore, identification and characterization of cellular genes that are targeted by oncogenic stimuli and in particular the potential roles of epithelial cell-specific components is likely to enhance our understanding of the molecular basis of breast cancer.

Recent molecular studies have focussed on the genetic lesions such as deletions, mutations and amplification of genes involved in the control of cell growth. These studies have identified the important roles of tumor suppressor, growth factor/growth factor receptor and proto-oncogene products in the genesis of cancers (2). In an attempt to define novel genes that are likely to play a role in breast and perhaps other epithelial cancers, we have established an in vitro model of MEC oncogenesis. Based on the epidemiologic evidence for the role of radiation in breast cancer (3-5), we exposed a normal MEC strain to fractionated doses of γ-irradiation which led to their tumorigenic transformation (6). The pair of normal (76N) and radiation-transformed tumorigenic MECs (76R-30) provided a novel system to identify genes whose expression was specifically altered during oncogenesis. Indeed, initial studies demonstrated a specific loss of the p53 tumor suppressor gene expression in 76R-30 cells as a result of the deletion of one allele and a small intronic deletion in the second allele which caused exon skipping (6).

In this proposal, we used subtractive hybridization between the 76R-30 and 76N cells to isolate a novel cDNA, designated NES1 (for normal epithelial specific-1), whose mRNA expression is dramatically down-regulated in radiation-transformed 76R-30 cells and is absent in most of established breast cancer cell lines. NES1 shows a high homology with members of three families of serine proteases: trypsin-like family, kallikrein family and activators of kringle domain-containing growth factors (7). Several of the serine proteases that show homology with NES1, such as the mouse nerve factor γ, the mouse epidermal growth factor binding protein, the human tissue plasminogen activator and the hepatocyte growth factor activators are known to convert inactive precursors of growth factors to mature active growth factors (8-12). The structural similarity of NES1 with cell-growth regulatory proteases and down-regulation of its mRNA expression in breast cancer cells suggest a potential role of this novel protein in the maintenance of the untransformed state of mammary and perhaps other epithelial cells.

PROPOSED SPECIFIC AIMS WERE

I. CHARACTERIZE THE EXPRESSION OF NES1 IN NORMAL AND TUMOR BREAST CELLS IN CULTURE AND IN TISSUE SPECIMENS.

1. Generate anti-NES1 antibodies.
2. Examine the expression of NES1 mRNA and protein in normal and tumor mammary cells in tissue sections and in cultures.
3. Examine the mechanisms of inducible NES1 expression.
4. Examine the effect of DNA damage on NES1 mRNA and protein expression.

II. ASSESS THE INFLUENCE OF ALTERATIONS IN NES1 EXPRESSION ON CELL GROWTH AND ONCOGENICITY.

1. Transfection of NES1 into mammary cells.
2. Growth properties and oncogenic behavior of NES1 transfectants.
3. Influence of reduced NES1 expression on growth, immortalization and oncogenicity of MECs.

III. CHARACTERIZE THE BIOCHEMICAL FUNCTIONS OF NES1.

1. Characterize the potential protease activity of NES1.
2. Examine non-protease biochemical functions of NES1 protein.
3. Mutational analysis of NES1 protein.

BODY OF THE REPORT

This section describes work accomplished from August 1996 to August 1997. First four sections (section 1 to 4) include results that were obtained after submission of this grant but were published (See Appendix Manuscript # 1 Liu et al., Cancer Research 56:3371-3379, 1996) before the funding initiated in August 1996. More recent results are described as sections 5 to 8.

1) Tissue-specific expression of NES1 mRNA (Aim I-2).

To assess the tissue distribution of NES1 expression, poly(A)$^+$RNA samples from various human tissues (Nylon purchased from Clontech) were hybridized with a NES1 probe corresponding to nucleotides 1-1069 of the full-length cDNA. As shown in Fig. 1, differential expression of the NES1 gene was observed in the tissues examined. Relatively abundant levels of the 1.4 kb mRNA transcript were observed in the prostate, testis, ovary, small intestine, colon, and lung with highest levels in the ovary. The pancreas showed abundant expression of a shorter (1.1 kb) mRNA transcript. In comparison, NES1 expression in thymus and heart was bearly detectable and was essentially undetectable in peripheral blood leukocytes, brain, placenta, lung, liver, skeletal muscle and kidney. Hybridization with the control probe 36B4 (13) demonstrated equal loading of mRNA in all lanes. These analyses show that NES1 mRNA is

expressed in a large subset of human tissues.

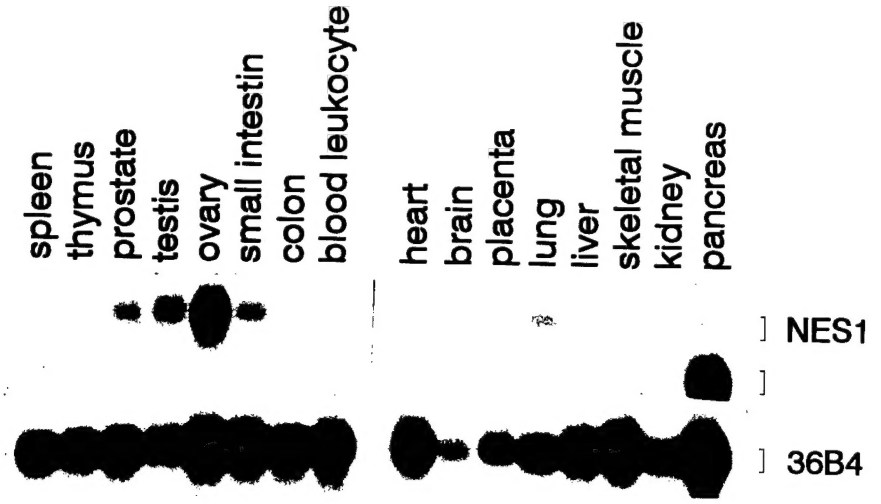

Fig. 1. Northern blot analysis of NES1 mRNA expression in various human tissues. Nylon membranes with 2 μg of poly(A)$^+$ RNAs from each of the indicated human tissues were obtained from Clontech, hybridized with a ^{32}P-labelled NES1 probe and visualized by autoradiography. 36B4 probe was used as a loading control.

2) Expression of NES1 in normal, immortalized and tumor mammary cells (Aim I-2).

As described above, NES1 cDNA was isolated by virtue of its drastically reduced mRNA expression in MECs oncogenically transformed by γ-irradiation. To further assess the relationship between NES1 mRNA expression and mammary tumor progression, we analyzed a number of normal MECs, mammary fibroblasts, immortalized MECs and established mammary tumor cell lines by Northern blot analysis. All of the normal (4 out of 4) mammary epithelial cells expressed abundant levels of 1.4 kb NES1 mRNA, whereas none of the fibroblast cell strains (5 out of 5) expressed detectable mRNA levels (hence the designation of Normal Epithelial Cell Specific-1 or NES-1) (Fig. 2 and data not shown). All of the MECs that have been induced to undergo pre-neoplastic transformation by HPV-16 E6 or E7 (10 out of 10) expressed NES1 mRNA levels similar to normal MECs (Fig. 2 and data not shown). In contrast, a drastic decrease or complete loss of NES1 mRNA was observed in all (16 out of 16) breast cancer cell lines examined (Fig. 2). Reprobing of the blot with a control probe 36B4 (13) showed that mRNA was indeed loaded in the lanes that showed little or no hybridization with NES1 probe (Fig. 2). These results show that while NES1 mRNA expression is unaffected by immortalization (preneoplastic transformation) of mammary cells, it is drastically down-regulated in a large subset of established breast cancer cell lines.

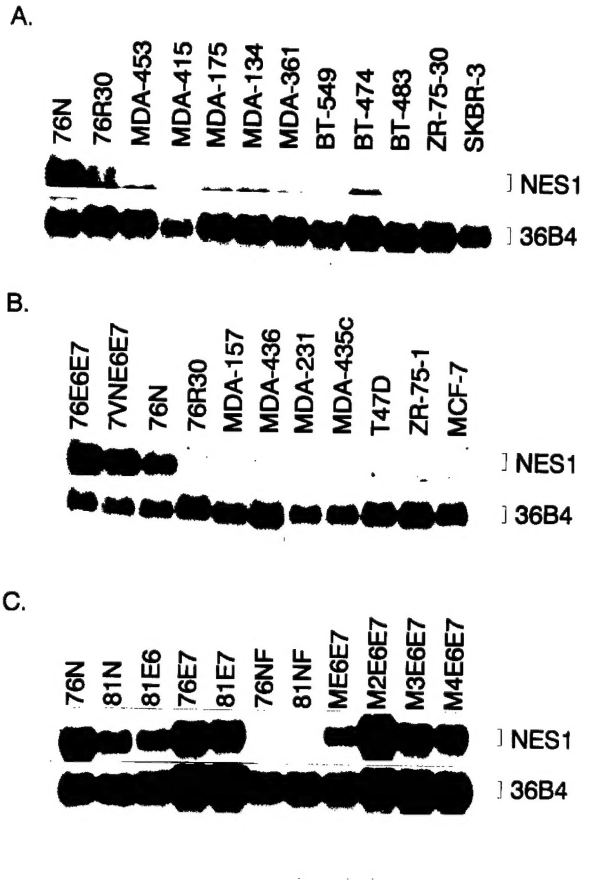

Fig. 2. Northern blot analysis of NES1 mRNA expression in cultured cell lines. Total cellular RNA (10 μg) from various cells was resolved on a 1.5 % agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with a 0.4-kb NES1 probe. Note a drastic decrease in NES1 mRNA levels in 76R-30 cells and almost complete loss in all mammary tumor cell lines. 36B4 was used as a loading control. A, B, and C are separate blots. A. 76N, normal MEC; 76R-30, radiation-transformed 76N; remaining cell lines represent breast cancer-derived cell lines. B. 76E6E7, 76N cells immortalized by HPV-16 E6+E7; 7VNE6E7, 7VN cells immortalized by HPV-16 E6+E7; 76N and 76R-30 as in A; remaining cell lines represent breast cancer-derived cell lines. C. First two lanes, 76N and 81N are two normal mammary epithelial cell strains; 81E6, a HPV-16 E6-immortalized 81N cells; 76E7 and 81E7 are HPV-16 E7-immortalized 76N and 81N cells respectively; 76NF and 81NF are two mammary fibroblast cell strains from two individuals designated 76 and 81 respectively; rest four lanes are HPV-16 E6+E7-immortalized human milk derived epithelial cell lines from three separate specimens.

3) Generation of anti-NES1 peptide antibody (Aim I-1).

Two NES1 peptides (a.a. 120-137, n..C-K-Y-H-Q-G-S-G-P-I-L-P-R-R-T-D-E-H-D..c; a.a. 38-50, n..Q-N-D-T-R-L-D-P-E-A-Y-G-A-P-C...c) were coupled to Keyhole limpet

hemocyanin through a C-terminal cysteine residue using maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL) for chemical cross-linking. The conjugates were used to immunize rabbits as a 1:1 emulsion with Titermax adjuvant (CYTRX Corporation, Georgia), as described earlier (14). Booster injections were given every 30 days and rabbits were bled 10 days after each injection. As shown below in Fig. 3, anti-peptide antibody (a.a 120-137) recognizes NES1 protein by Western blot analysis. Similar results were obtained with second anti-peptide antibody (see Fig. 5).

4) NES1 is a secreted protein (Aim I-2).

Most of the proteins that showed homology to predicted NES1 polypeptide contain a signal peptide at the N-terminus (first 14 to 15 amino acids after methionine) and are secreted. Although the N-terminus of NES1 did not show a significant homology to corresponding region of the other serine proteases. This region was strongly hydrophobic (see manuscript, Liu et al., 1996), consistent with its being a signal peptide and suggestive of NES1 being a secreted protein. To examine this possibility, we used a rabbit antiserum against a NES1-specific peptide (a.a. 120-137). This antiserum was used for Western blot analysis of cell lysates and culture supernatants derived from NES1 mRNA-positive and -negative MECs (Fig. 2). As seen in Fig. 3, anti-NES1 antibodies specifically detected a 30 kDa polypeptide almost exclusively in the culture supernatants of all of the NES1 mRNA-positive cell lines. The 30 kDa polypeptide was not detected in the NES1 mRNA-negative cell lines. These data strongly support the possibility that NES1 is a secreted protein similar to other homologous serine proteases.

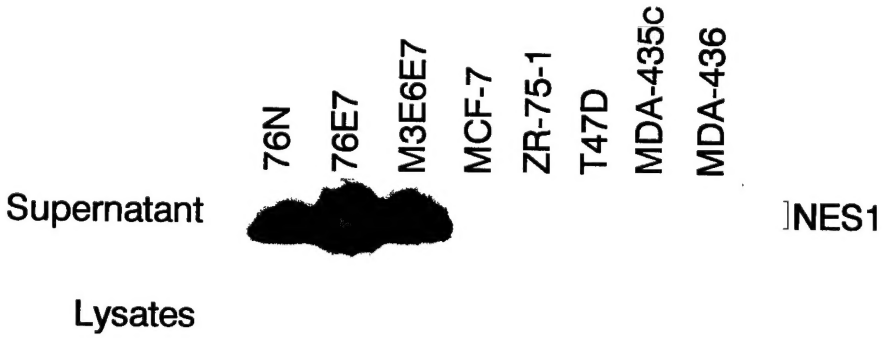

Fig. 3. Western blot analysis of NES1 protein. Aliquots of concentrated culture supernatant or cell lysates derived from number of indicated cell lines were resolved by a SDS 10 %-PAGE and transferred to PVDF membrane. Membranes were immunoblotted with anti-NES1 antiserum followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence.

5) Transfection of NES1 cDNA into NES1-negative mammary tumor cell lines (Aim II-1).

A full-length NES1 cDNA was cloned into pCMVneo vector. The NES1 expression construct was transfected into MDA-MB-231 mammary cancer cell line by a calcium phosphate method. Briefly, $5X10^5$ cells per 100-mm-diameter dish were plated in α-MEM containing 10% FCS, 18 hours prior to transfection. Linearized plasmid DNA (8 μg) was mixed with calcium phosphate to prepare a coprecipitate which was added to cells. After 6 hours, cells were treated with 15 % (vol/vol) glycerol for 4 min and fresh medium was added. At 48 hour after transfection, G418 selection (1 mg/ml; GIBCO) was initiated. After about 2 weeks, G418-resistant colonies were seen. Once the colonies attained the size of about 100 cells, they were picked using cloning cylinders and cultured separately. Stably transfected clones were examined for expression of NES1 mRNA by Northern blotting with NES1 probe and secretion of NES1 protein into culture supernatants by immunoblotting with anti-NES1 antibody. Four clones (# 2,4,5, and 6) out of six showed high levels of NES1 mRNA (Fig. 4) and the same clones secreted high levels of NES1 protein in culture medium (Fig. 5).

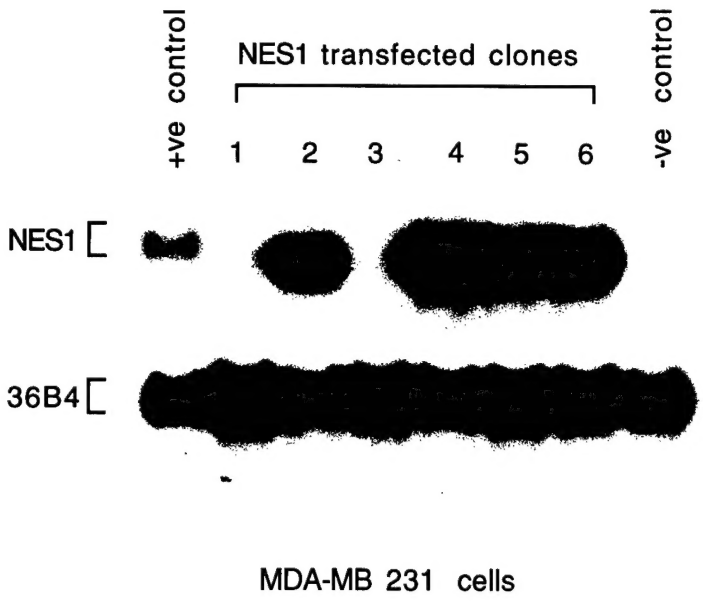

Fig. 4. Northern blot analysis of NES1 mRNA expression in various clonal NES1-transfectants of MDA-MB-231 cell line. Total cellular RNA (10 μg) from various clones was resolved on a 1.5 % agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with a ^{32}P-labelled 0.4-kb NES1 probe followed by autoradiography. Note that clones #s 2,4,5 and 6 express high levels of NES1 mRNA. Hybridization to 36B4 probe (bottom panel) was used as a loading control.

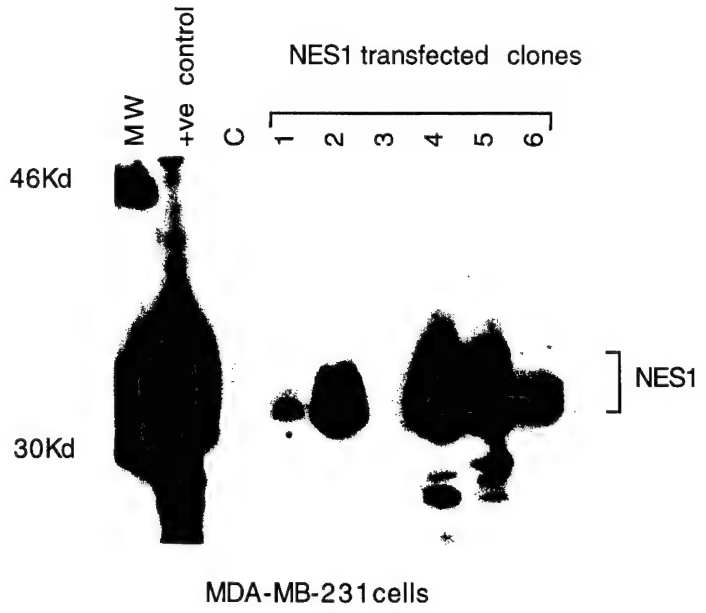

Fig. 5. Protein analysis in the supernatants of NES1-transfected clones of MDA-MB-231 using Western Blot Analysis. Control or NES1-transfectants were grown to 80-90% confluence in 25 cm^2 flasks. Cells were then washed once with serum-free medium and further cultured in this same medium for 20 h. Supernatant from each flask was concentrated to 150 μl using a 10 kDa cut-off filter (Centricon, Amicon Inc., MA), 50 ug proteins of concentrated supernatants was mixed with an equal volume of 2X sample buffer, was resolved on SDS 10%-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, MA) as described earlier (15). Membranes were incubated with anti-NES1 antisera followed by goat anti-rabbit IgG conjugated to horse-radish peroxidase (Pierce, Rockford, IL). Enhanced chemiluminescence (ECL) detection was performed according to manufacturer's instructions (Amersham, Arlington Heights, IL). Fig. 5 shows that NES1 protein secretion was observed in the same clones that expressed the NES1 mRNA (Fig. 4).

6) NES1 inhibits anchorage-independent growth (Aim II-3).

We examined the ability of untransfected and NES1-transfected cells to exhibit anchorage-independent growth. This property correlates with oncogenic behavior of mammary cells and thus serves as an indicator of altered oncogenicity of transfectants. Based on our hypothesis, we anticipated that the transfected cells may show reduced anchorage-independent growth.

As shown in Fig. 6 both NES1-expressing clones tested (clone # 2 and 4) dramatically reduced anchorage-independent growth.

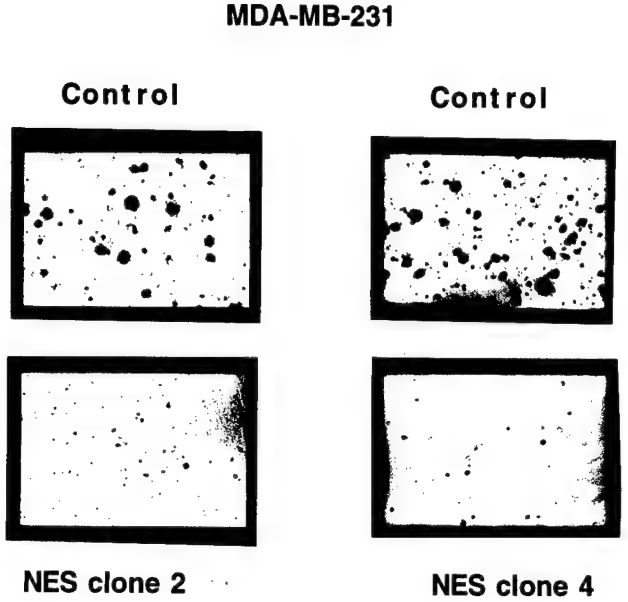

Fig 6. Comparison of anchorage-independent growth of control MDA-MB-231 cells with NES1-transfectants. Cells (5 X 10^4/60-mm dish) were plated in a top layer of 0.3 % agar with a bottom layer of 0.5 % agar (1:1 in 2X medium). Colonies were photographed after 2 weeks to provide an assessment of anchorage-independent growth. Note that both NES1-transfectants had dramatic reduction in colony formation in soft agar as compared to control cells.

In view of dramatic reduction in anchorage-independent growth of MDA-MB-231 cells upon NES1 overexpression, we transfected NES1 into two other breast cancer cell lines MDA-MB-435 and MDA-MB-468. Both of these cell lines are known to be highly tumorigenic when implanted in the mammary fat pad of the nude mice, and this would allow an assessment of the effect of NES1 expression on tumorigenicity. Fig. 7 and 8 show NES1 protein expression in NES1 transfectants of MDA-MB-435 and MDA-MB-468 respectively. In each case, several clonal transfectants expressing NES1 mRNA and secreting NES1 protein into culture medium were obtained. These transfectants will be analyzed in vitro in soft agar cloning assay as well as in the nude mouse assay. Together these in vitro and in vivo assay systems will allow us to assess the influence of NES1 expression on cell growth and oncogenic behavior.

Fig. 7

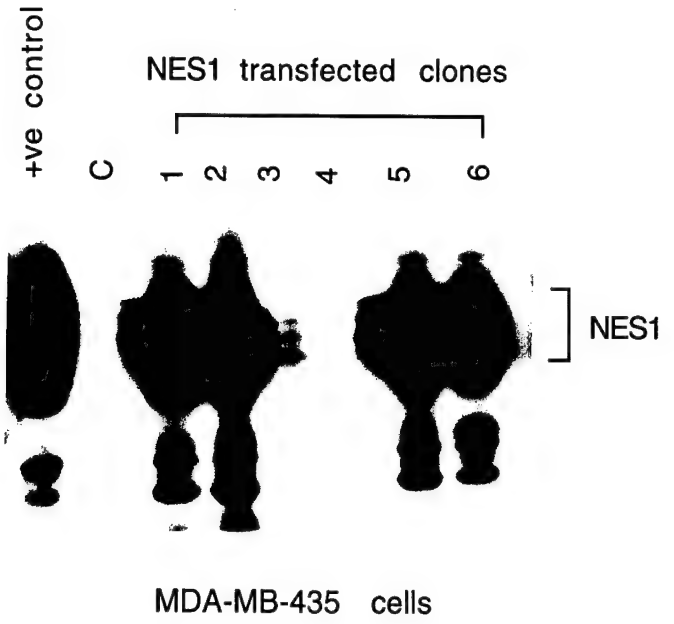

Fig. 8

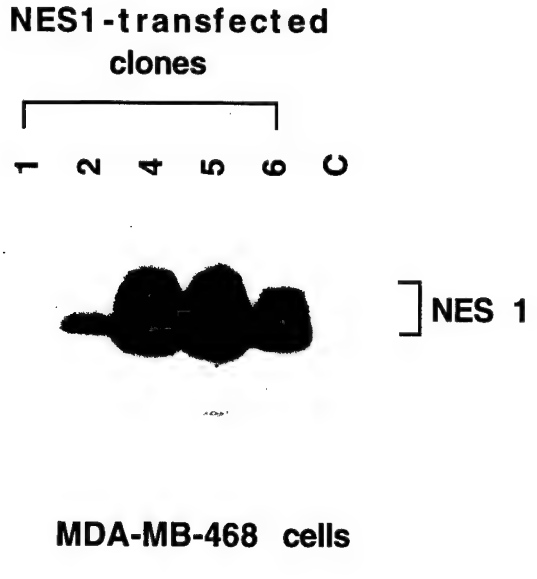

Fig. 7 and 8. Protein analysis in the supernatants of NES1-transfected clones of MDA-MB-435 and MDA-MB-468 by Western Blot Analysis. Control or NES1-transfectants were grown to 80-90% confluency in 25 cm^2 flasks in α-MEM containing FCS. Cells were then washed once with a serum-free medium and further cultured in this medium for 20 h. 50 μg of protein from unconcentrated supernatants were used to analyze in Western blot as described in Fig. 5. Fig. 7 shows 4 out of 6 clones (clone # 1, 2, 5 and 6) expressing high levels of NES1 protein in MDA-MB-435 cells. Fig. 8 shows 3 (clone # 4, 5, and 6) out of 5 clones expressing high levels of NES1 protein. All clones that show high levels of NES1 protein in Figs. 7 and 8 also showed high levels of mRNA (data not shown).

7) Expression of an epitope-tagged NES1 protein in insect cells (Aim III-1).

Using the polymerase chain reaction (PCR), we have generated cDNA expression plasmids that are predicted to append an epitope tag for anti-myc antibody (9E10) at the N- or C- terminus of NES1 protein [16]. Tagged and untagged NES1 constructs were cloned into pBacPAK6 baculovirus vector (Clontech) and introduced into insect cells (IPLB-Sf21). Expression of 3' and 5' tagged NES1 was demonstrated using immunoblotting of the insect cell supernatants with anti-myc and anti-NES1 antibodies. As shown in Fig. 9, both 3' and 5'myc tagged NES1 were expressed and secreted into culture supernatant of infected insect cells.

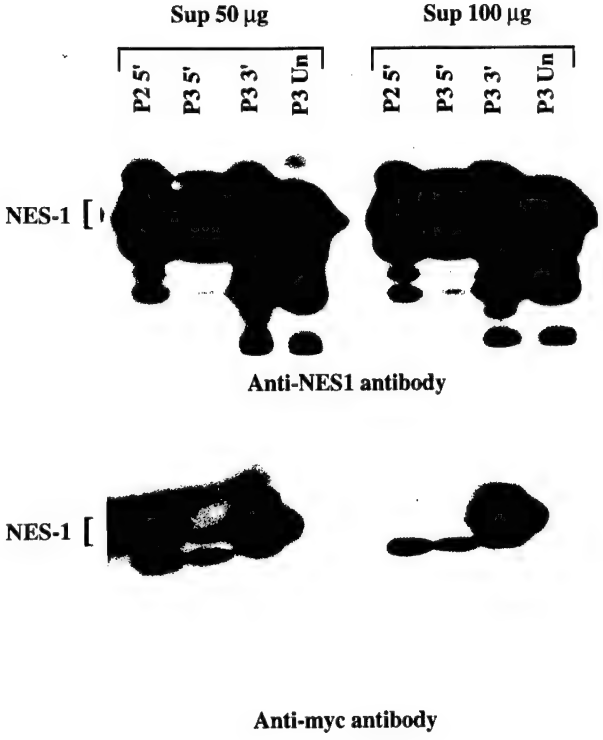

Fig. 9. Western blot analysis of NES1 protein in culture supernatants of insect cells transfected with untagged or myc epitope-tagged NES1. Untagged or myc-epitope tagged NES1 constructs were transfected into insect (Sf21) cells by calcium phosphate method. Sf21 cells were propagated as monolayer in T150 flasks in Grace's insect cell medium supplemented with yeastolate, lactalbumin hydrolysate and 10% fetal calf serum at 27°C without CO_2. Upon reaching 60-70% confluency cells were infected with passage 2 or passage 3 virus expressing untagged NES1 or 5' or 3'myc-tagged NES1 protein. After 48 hours of infection, the medium was collected. 50 or 100 μg of protein from unconcentrated culture supernatants was resolved by a SDS-10 %-PAGE and transferred to a PVDF membrane. Membranes were immunoblotted with anti-NES1 antiserum (above panel) or anti-myc (lower panel) mAb (9E10) followed by goat anti-rabbit IgG or rabbit-anti-mouse conjugated to horse-radish peroxidase. Detection was by enhanced chemiluminescence. Note that immunoblotting of supernatants with anti-myc antibody (9E10) and anti-NES1 antibody shows NES1 proteins of expected size (30 kDa) with both 3' and 5' tagged constructs. Supernatants from cells transfected with untagged NES1 served as control (lower panel). Immunoblotting of supernatants with NES1 antiserum shows NES1 protein of expected size in all untagged as well as 3' and 5' tagged construct transfected cells (above panel).

8) Purification of NES1 protein (Aim III).

The results presented above demonstrated that we had successfully expressed NES1 as a C-and N- terminal myc-tagged protein in Sf21 insect cells (Fig. 9). This system is known to allow proteins to undergo posttranslational modifications in eukaryotic cells and yields large quantities of enzymatically active proteins in numerous situations. As shown in Fig. 9, Sf21 cells expressed and secreted large quantities of NES1 protein. For purification of NES1 protein five T150 flasks of sf21 cells were infected with third progeny of BacPAK6 cells transfected with 3'-myc tagged NES1 (titre about $2X10^8$ Pfu/ml). 48 hours post-infection, the medium was collected. To prevent metal ion catalyzed oxidation of proteins, EDTA was added to a final concentrations of 1 mM. Protein concentration, measured was 2.38 mg/ml (total protein was 587 mg). Ammonium sulphate was added to give 25 % saturation (144 g/litre). Precipitate was dissolved in a buffer containing 350 mM NaCl and 10 mM Tris, pH 7.4. Volume of the supernatant was measured and 429 g/litre ammonium sulphate was added to give a final saturation of 85%. The 85% ammonium sulfate precipitate was dissolved in 350 mM NaCl and 10 mM Tris and was dialyzed in 10,000 M.W. cutoff dialysis tubing against 10 mM Tris and 150 mM NaCl to remove excess salts. The unconcentrated supernatant (control lane 1), 25 % precipitates, 85 % precipitates and 85 % supernatant were analyzed for the presence of NES1 by Western blotting. The amount of protein in 25 % ammonium sulfate precipitate was 1.92 mg (0.3 % of original amount) as compared to 49.68 mg (8.5 % of original amount) in 85 %. Therefore, for further studies 85 % ammonium sulfate precipitate was used.

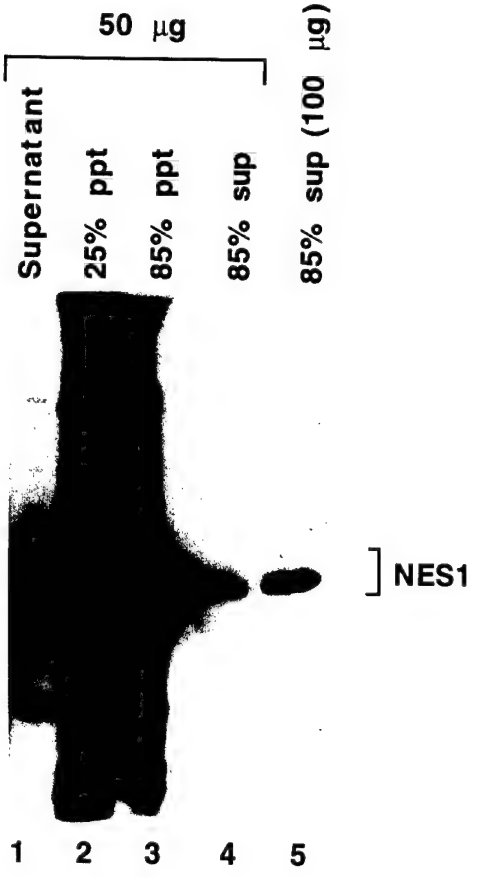

Fig. 10. Western blot analysis of NES1 protein in supernatants of insect cells transfected with 3'-myc tagged NES1. Cells were grown, their supernatant were immunoblotted with anti-NES1 antibody as described in Fig. 9. Lane 1, NES1 protein in supernatants collected from virus infected Sf21 cells before ammonium sulphate fractionation; Lanes 2 and 3, NES1 in precipitate at 25 % and 85 % saturation of ammonium sulfate. Lanes 4 and 5 show residual NES1 protein in 50 µg or 100 µg protein samples of the supernatant remaining after 85 % ammonium sulfate precipitation. Lanes 1-4 were loaded with 50 µg of protein samples. Although 25 % and 85 % ammonium sulfate precipitate when loaded equally show similar quantity of NES1 protein, the total amount of protein with 25 % precipitate was 26 fold lower than in 85 % precipitate.

The 85 % ammonium sulfate precipitate of the above Sf21 culture supernatant was loaded onto a 40X2.5 cm SP-Sepharose fast flow ion exchange column at a flow rate of 3 ml/min. The column was equilibrated with 50 mM sodium phosphate buffer, pH 7.2. After washing with four volumes of buffer, the bound proteins were eluted with a linear gradient of 1 M KCl. O.D. at 280 nm was monitored on fractions using an attached UV monitor. The plot of the UV absorbance (Fig. 11) showed that the bound proteins eluted as a broad peak over 90 minutes. 6 ml fractions were collected and analyzed by immunoblotting with anti-NES1 antibody (Fig. 12). NES1 was detected in fractions 16-20 out of 25 fractions analyzed. Fractions corresponding to peak NES1 immunoreactivity (17-19) and 2 surrounding fractions (18 and 20) were concentrated using centricon 10 membrane (molecular weight cut off 10,000). 40 μg protein samples of concentrated fractions were run on 10% SDS-PAGE and protein bands were localized by silver staining (Fig. 13). Silver staining showed that the peak NES1 fractions are relatively free of contaminating protein. Second peak after NES1 peak when tested in Western blot as well as silver staining showed no detectable NES1 protein (data not shown).

This methodology will be further improved by altering the slope of the gradient used for elution, by changing flow rate and altering other parameters. In addition, future experiments will use serum-free medium while collecting supernatant from insect cells for purification studies. Serum-free medium sf900II, is already purchased from Life Technologies, Grand Island, New York.

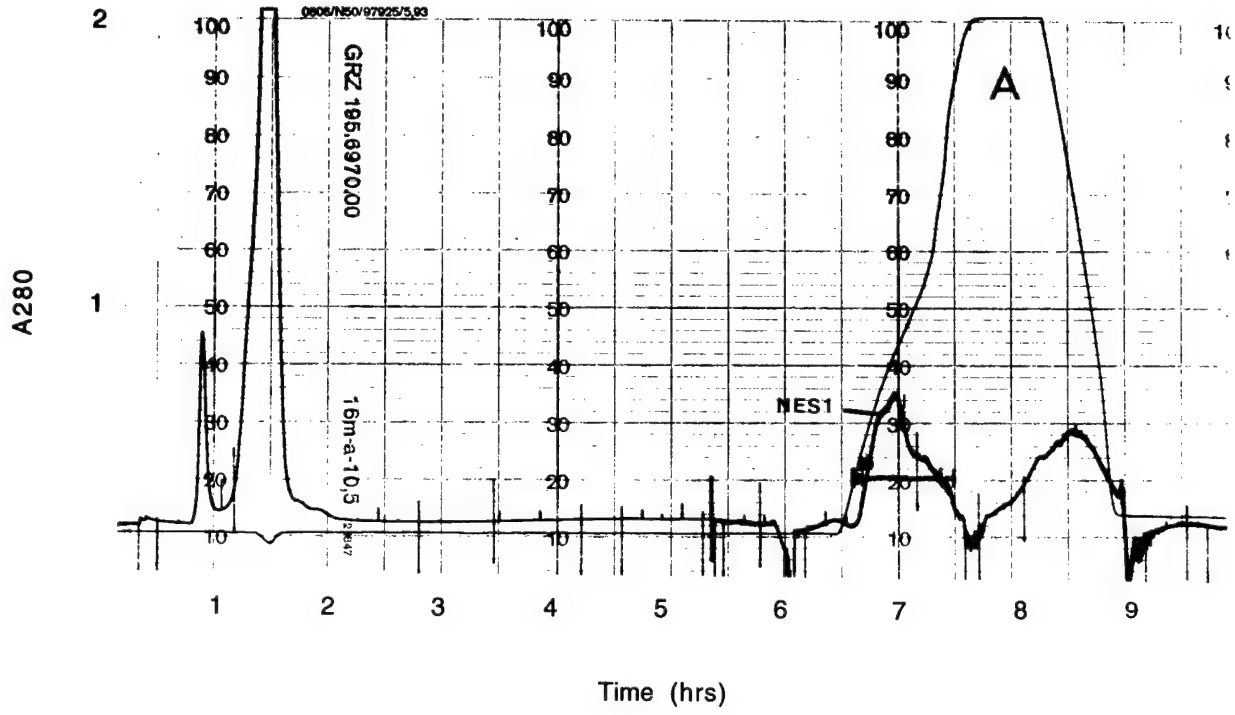

Fig. 11. Elution profile of the polypeptides bound to SP sepharose fast flow column loaded with 85 % ammonium sulfate precipitate of NES1 containing Sf21 cell supernatant. 40 X 2.5 cm column was loaded with 10 mg of 85 % ammonium sulfate precipitate of Sf21 cells infected with 3'myc-tagged NES1. The column was washed with four volumes of phosphate buffered saline (over 6 hours) and eluted with a salt gradient (0-1 M KCl in 50 mM $NaPO_4$). 45 fractions between hour 6 to 7:30 containing the peak O.D. 280 were processed further for anti-NES1 immunoblotting (Fig. 12). Peak labelled 'A' depicts linearity of the gradient.

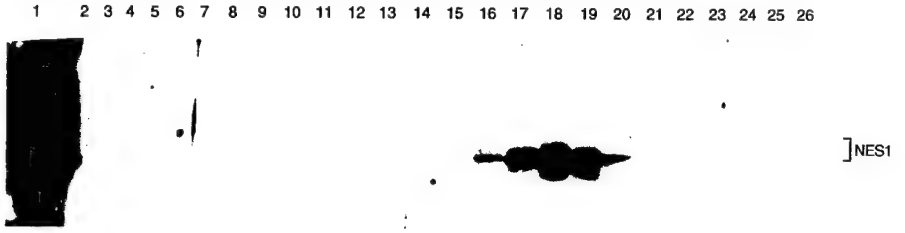

Fig. 12. Western blotting of the column fractions. 100 μl of each fraction (45 fractions, two fractions pooled into one, labelled as fraction # 4-26) was run on 10 % SDS PAGE, immunoblotted with anti-NES1 antibody and processed for Western blot as above. Fraction 1, positive control; Fractions 16-20, peak of NES1 (as indicated). Note that only ten fractions (16-20) showed NES1 protein.

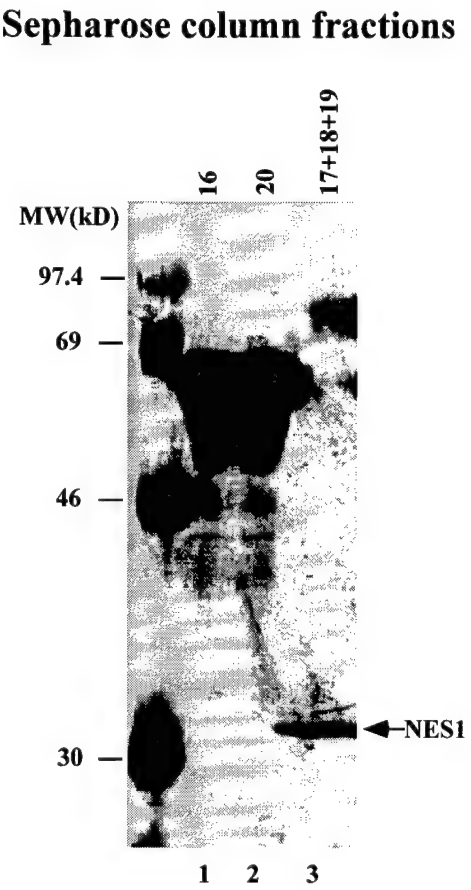

Fig. 13. Silver staining of fractions 16-20 eluted from SP-Sepharose column by KCl. As shown in Fig. 12 fractions 17,18 and 19 showed majority of NES1 protein. These fractions were pooled together, and fraction 16 and 20 were used separately. All of these fractions were concentrated 10 fold using centricon filter 10 (M.W. cutoff of 10,000) and then 40 μg protein samples of each were loaded on a 10 % SDS-PAGE followed by silver staining as described in ref. 17. Note that pooled fractions 17,18 and 19 together showed a major band of 30 kDa corresponding to the NES1 protein, whereas fraction 16 and 20 showed primarily the contaminated proteins and undetectable NES1 protein. An higher molecular band in lane 3 may be due to FCS in the supernatant of insect cells. We are currently using commercially available serum-free medium instead of regular FCS-containing medium for purification studies.

Summary and Conclusions.

In an effort to isolate genes whose expression at the mRNA level is down-regulated during oncogenic transformation of human mammary epithelial cells (MECs), we performed subtractive hybridization between a normal MEC strain 76N and its radiation-transformed tumorigenic derivative 76R-30. We have isolated cDNA clones corresponding to a 1.4 kb mRNA species that is abundantly expressed in 76N cells but is drastically reduced in 76R-30 cells. Based on its selective expression in mammary epithelial compared to fibroblast, the corresponding gene is designated as normal epithelial specific-1 (NES1). Sequence analysis of the full length NES1 cDNA clones revealed it to be a novel gene with a predicted polypeptide of 30.14 kDa; in vitro transcription and translation confirmed this prediction. Data-base searches revealed a 50-63 % similarity and 34-42% identity with several families of serine proteases, in particular the trypsin-like proteases, members of the glandular kallikrein family (including the prostate specific antigen, the nerve growth factors-γ, and epidermal growth factor binding protein) and the activators for the kringle family proteins (including the human tissue plasminogen activator and the human hepatocyte growth factor activator). Importantly, all of the residues known to be crucial for substrate binding, specificity and catalysis by the serine proteases are conserved in the predicted NES1 protein, suggesting that it may be a protease. An anti-peptide antibody directed against a unique region of the NES1 protein (a.a. 120-137) detected a specific 30 kDa polypeptide almost exclusively in the supernatant of the mRNA-positive MECs, suggesting that NES1 is a secreted protein. The 1.4 kb NES1 mRNA was expressed in several organs (thymus, prostate, testis, ovary, small intestine, colon, heart, lung and pancreas) with highest levels in ovary; a 1.1 kb transcript was found in the pancreas. While expression of the NES1 mRNA was observed in all normal and immortalized non-tumorigenic MECs, the majority of human breast cancer cell lines showed a drastic reduction or a complete lack of its expression. Transfection of NES1 cDNA into a mammary tumor cell line MDA-MB-231 showed dramatic reduction in anchorage-independent growth. The structural similarity of NES1 to polypeptides known to regulate growth factor activity and a negative correlation of NES1 expression with breast oncogenesis suggest a direct role for this novel protease-like gene product in the suppression of tumorigenesis.

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Identification of a Novel Serine Protease-like Gene, the Expression of Which Is Down-Regulated during Breast Cancer Progression[1]

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ABSTRACT

In an effort to isolate genes with down-regulated expression at the mRNA level during oncogenic transformation of human mammary epithelial cells (MECs), we performed subtractive hybridization between normal MEC strain 76N and its radiation-transformed tumorigenic derivative 76R-30. Here, we report the isolation of cDNA clones corresponding to a 1.4-kb mRNA species that is abundantly expressed in 76N cells but is drastically reduced in 76R-30 cells. Based on its selective expression in MECs compared with fibroblasts, the corresponding gene is designated *NES1* (normal epithelial cell-specific 1). Sequence analysis of the full-length NES1 cDNA clones revealed it to be a novel gene with a predicted polypeptide of 30.14 kilodaltons; *in vitro* transcription and translation confirmed this prediction. Database searches revealed a 50–63% similarity and 34–42% identity with several families of serine proteases, in particular the trypsin-like proteases, members of the glandular kallikrein family (including prostate-specific antigen, nerve growth factor γ, and epidermal growth factor-binding protein) and the activators for the kringle family proteins (including the human tissue plasminogen activator and human hepatocyte growth factor activator). Importantly, all of the residues known to be crucial for substrate binding, specificity, and catalysis by the serine proteases are conserved in the predicted NES1 protein, suggesting that it may be a protease. An antipeptide antibody directed against a unique region of the NES1 protein (amino acids 120–137) detected a specific 30-kilodalton polypeptide almost exclusively in the supernatant of the mRNA-positive MECs, suggesting that NES1 is a secreted protein. The 1.4-kb NES1 mRNA was expressed in several organs (thymus, prostate, testis, ovary, small intestine, colon, heart, lung, and pancreas) with highest levels in the ovary; a 1.1-kb transcript was found in the pancreas. Although expression of the NES1 mRNA was observed in all normal and immortalized nontumorigenic MECs, the majority of human breast cancer cell lines showed a drastic reduction or a complete lack of its expression. The structural similarity of NES1 to polypeptides known to regulate growth factor activity and a negative correlation of NES1 expression with breast oncogenesis suggest a direct or indirect role for this novel protease-like gene product in the suppression of tumorigenesis.

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths of women in North America and Europe. About 180,000 new cases of breast cancer are diagnosed every year in the United States alone, with one in eight women estimated to develop breast cancer during her lifetime. In nearly all cases, the etiology of these cancers is unknown. Similar to other human cancers, the vast majority of breast cancers represents a malignant transformation of epithelial cells. Malignant transformation represents a complex, multistep process in which genetic changes and environmental factors, such as radiation, viruses, and dietary factors, are thought to deregulate the cellular processes that control cell proliferation and differentiation (1). Therefore, identification and characterization of cellular genes that are targeted by the oncogenic stimuli and, in particular, the potential roles of epithelial cell-specific components is likely to enhance our understanding of the molecular basis of breast cancer.

Recent molecular studies have focussed on genetic lesions, such as deletions, mutations, and amplification of genes, involved in the control of cell growth. These studies have identified the important roles of tumor suppressor, growth factor, growth factor receptor, and proto-oncogene products in the genesis of cancers (2). In an attempt to define novel genes that are likely to play a role in breast and perhaps other epithelial cancers, we have recently established an *in vitro* model of MEC[3] oncogenesis. Based on the epidemiological evidence of the role of radiation in breast cancer (3–5), we exposed a normal MEC strain to fractionated doses of γ-irradiation, which led to its tumorigenic transformation (6). The pair of normal (76N) and radiation-transformed tumorigenic (76R-30) MECs provided a novel system to identify genes with specifically altered expression during oncogenesis. Indeed, initial studies demonstrated a specific loss of *p53* tumor suppressor gene expression in 76R-30 cells as a result of the deletion of one allele and a small deletion in the second intronic allele, which caused exon skipping (6).

Here, we used subtractive hybridization between the 76R-30 and 76N cells to isolate a novel cDNA, designated NES1 (for normal epithelial cell specific 1), the mRNA expression of which is dramatically down-regulated in radiation-transformed 76R-30 cells and absent in most of established breast cancer cell lines. NES1 shows a high homology with the families of members of three serine proteases: the trypsin-like family, kallikrein family, and activators of kringle domain-containing growth factors (7). Several of the serine proteases that show homology with NES1, such as mouse nerve factor γ, mouse EGF-binding protein, human tissue plasminogen activator, and hepatocyte growth factor activators, are known to convert inactive precursors of growth factors to mature, active growth factors (8–12). The structural similarity of NES1 with cell growth regulatory proteases and down-regulation of its mRNA expression in breast cancer cells suggest a potential role of this novel protein in the maintenance of the untransformed state of mammary and perhaps other epithelial cells.

MATERIALS AND METHODS

Sources of Cells. Normal mammary epithelial cells (81N and 76N), fibroblast cells (76NF and 81NF), radiation-transformed cells (76R-30), HPV-16 E6 and/or E7-immortalized epithelial cells (81E6, 76E7, 81E7, 76E6E7, and 7VNE6E7), and milk-derived epithelial cells-immortalized with HPV-16 E6 and E7 (ME6E7, M2E6E7, M3E6E7, and M4E6E7) were established in our laboratory (6, 13, 14). The MCF-7 cell line was obtained from the Michigan Cancer Foundation. All other breast tumor cell lines (MDA-MB-134, MDA-MB-157, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-435C, MDA-MB-436, MDA-MB-453, BT-474, BT-483, BT-549, ZR-75-1, ZR-75-30, T-47D, and SK-BR-3) were obtained from the American Type Culture Collection.

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[3] The abbreviations used are: MEC, mammary epithelial cell; HPV, human papillomavirus; poly(A)+, polyadenylated; oligo(dT), oligodeoxythymidylic acid; kDa, kilodalton; NGF, nerve growth factor; EGF, epidermal growth factor.

Cell Culture Conditions. Normal and immortal mammary epithelial cells were maintained in DFCI-1 medium (13). Immortalized milk-derived epithelial cells were grown in DFCI-1 medium containing 10% FCS (14). All tumor cell lines were grown in α-MEM supplemented with 10% FCS, as described earlier (15).

Construction of cDNA Libraries. A subtraction cDNA library was custom made through Novagen (Madison, WI). Briefly, a cDNA library was first prepared by reverse transcription from 76R-30 poly(A)$^+$ RNA using an oligo(dT)-*Hin*dIII primer adapter, klenow-mediated second-strand cDNA synthesis, attachment of *Eco*RI linkers, and directional cloning into *Eco*RI-*Hin*dIII sites of the λSHlox vector (16). Phage DNA was prepared from the 76R-30 cDNA library, digested with *Not*I, and *in vitro* transcribed with the T7 RNA polymerase to yield the "driver" cRNA for subtraction. cRNA was biotinylated using photoprobe biotin (17). For subtraction, ^{32}P-labeled first-strand cDNA was prepared from 76N poly(A)$^+$ RNA by reverse transcription. This cDNA was subjected to two rounds of subtractive hybridization with a 10-fold excess of biotinylated 76R-30 driver cRNA (16, 17). Hybridization was prepared in the presence of poly(A)$^+$ oligomers to prevent oligo(dT)/oligo(dA) interactions between the cRNA and cDNA. After each round, excess driver cRNA and double-stranded hybrids were removed with streptavidin and phenol. This procedure resulted in the depletion of 99.54% of 76N cDNA. The remaining single-stranded 76N cDNA was used for second-strand cDNA synthesis using Klenow; *Eco*RI adapters were ligated to ends; and cDNA was cloned into the *Eco*RI and *Hin*dIII sites of the λSHlox vector. This cDNA library is referred to as the 76NS library. Recombinant phages were plated at low density, and single plaques were picked and converted into the plasmid form by Cre-recombinase-mediated autosubcloning (16). The plasmid DNA was isolated from individual colonies and digested with *Eco*RI and *Hin*dIII. Isolated *Eco*RI-*Hin*dIII inserts were ^{32}P labeled and used as probes for Northern blot hybridization to poly(A)$^+$ mRNA isolated from the 76N and 76R-30 cells.

A second 76N cDNA library in the pGAD10 vector was custom constructed through Clontech (Palo Alto, CA). Here, the first-strand cDNA was synthesized using oligo(dT) plus random primers, followed by DNA polymerase 1-mediated second-strand synthesis, addition of *Eco*RI adapters, and cloning into the *Eco*RI site of the PGAD10 vector (18). This library was screened with ^{32}P end-labeled oligonucleotides (see "Results").

Sequencing of the *NES1* Gene. pSHlox-1 plasmid recombinants containing NES1 cDNA inserts were used as templates for double-strand sequencing using Sequenase (United State Biochemical Corp., Cleveland, OH). Sequences near the ends of cDNA inserts were obtained with the following primers corresponding to flanking the vector sequences. pSHlox vector primers were: T7 promoter primer, 5′-GGC-CTC-TAA-TAC-GAC-TCA-C-3′; and SP6 promoter primer, 5′-CCG-CAG-ATT-TAG-GTG-ACA-C-3′. PGAD10 primers were: an upstream primer, 5′-TAC-CAC-TAC-AAT-GGA-TG3′; and a downstream primer, 5′-GTT-GAA-GTG-AAC-TTG-CGG-GC3′. The remaining part of the NES1 inserts was sequenced using 12 sense strand (nucleotides 6–22, 72–91, 128–145, 196–213, 344–360, 484–500, 634–650, 723–739, 851–867, 998-1116, 1125–1141, and 1253–1269) and 10 antisense strand (nucleotides 1392–1377, 1294–1277, 1201–1185, 1086–1069, 917–899, 807–789, 674–657, 516–488, 292–275, and 176–161) NES1-specific primers.

***In Vitro* Translation of NES1.** An NES1 cDNA fragment containing nucleotides 1–1069 (see Fig. 2), which include the predicted polypeptide-coding region, was cloned in the pBluescript II KS vector (Stratagene, La Jolla, CA) in both sense and antisense orientations, and plasmid DNA was used for coupled *in vitro* transcription and translation, using a commercial kit (Promega, Madison, WI), as described (19). [^{35}S]methionine was incorporated during translation to allow visualization of the synthesized polypeptide by SDS-PAGE followed by fluorography.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from 50–70% confluent cell monolayers using the guanidium-isothiocyanate method (20). Northern blot hybridization was carried out using a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL) and ^{32}P-labeled cDNA insert (nucleotides 651-1072), as described previously (21). Nylon membranes with poly(A)$^+$ RNAs from various human tissues (tissue blots) were obtained from Clontech.

Southern Blot Analysis. Ten μg genomic DNA were digested to completion with restriction enzymes (Life Technologies, Inc., Gaithersburg, MD), resolved on agarose gels, transferred to a Hybond-N nylon membrane, and hybridized with a ^{32}P-labeled NES1 cDNA probe corresponding to nucleotides 1–1069, as described earlier (21).

Generation of Anti-NES1 Antibody. A NES1 peptide (amino acids 120–137, n..C-K-Y-H-Q-G-S-G-P-I-L-P-R-R-T-D-E-H-D..c) was coupled to keyhole limpet hemocyanin through a C-terminal cysteine residue using maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) for chemical cross-linking. The conjugate was used to immunize rabbits as a 1:1 emulsion with Titermax adjuvant (CytRx Corp., Norcross, Georgia), as described earlier (22). Booster injections were given every 30 days, and rabbits were bled 10 days after each injection.

Western Blot Analysis. Normal, immortalized, and tumor mammary cells were grown to 80–90% confluency in their respective media in 25-cm^2 flasks. Cells were then washed once with a serum and bovine pituitary extract-depleted DFCI-1 medium (D2) as described earlier (21) and then further cultured in this medium for 20 h. Supernatants were collected, and cells were lysed in SDS-PAGE sample buffer. The supernatant from each flask was concentrated to 150 μl using a 10-kDa cutoff filter (ultrafree centrifugal filter device; Millipore, Marlborough, MA) and mixed with an equal amount of 2× sample buffer. Concentrated supernatant and lysates representing an equal number of cells were resolved on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) as described earlier (14). Membranes were incubated with anti-NES1 antisera followed by goat antirabbit IgG conjugated to horseradish peroxidase (Pierce). Enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Amersham).

RESULTS

Isolation of a cDNA Clone Corresponding to an mRNA Expressed in 76N Cells but Markedly Down-Regulated in the 76R-30 Radiation-transformed Cell Line. A cDNA library was prepared from the poly(A)$^+$ RNA of the 76N normal MEC strain after the RNA messages cross-hybridizing with its radiation-transformed derivative 76R-30 strain (6) were depleted by two rounds of subtractive hybridization (see "Materials and Methods"). This resulted in the removal of 99.54% of the total cDNAs, suggesting that the remaining cDNAs were likely to be enriched for the mRNA species that are preferentially expressed in the 76N cells. Single bacteriophage plaques from this library (76NS) were converted into plasmid form by Cre-mediated recombination, and their cDNA inserts were cleaved with *Eco*RI and *Hin*dIII (restriction enzymes used for cloning; Refs. 16 and 17). Isolated cDNA inserts were labeled with [^{32}P]dCTP and [^{32}P]dGTP and used as probes in Northern blotting to detect the relative mRNA expression in 76N and 76R-30 cells. One cDNA insert (clone 37, about 0.4 kb) hybridized to an abundant 1.4-kb transcript in 76N normal parental cells (Fig. 1). In contrast, 76R-30 cells showed dramatically reduced expression of this mRNA. Thus, clone 37 represented a gene with expression that appeared to be down-regulated at the mRNA level during oncogenic transformation of the 76N MECs. As described below, mRNA corresponding to cDNA clone 37 is expressed in MECs but not in mammary fibroblasts. Therefore, the corresponding gene has been designated normal epithelial cell-specific 1 or *NES1*.

Sequence Analysis of NES1 cDNA Reveals It to Be a Novel Serine Protease-like Gene. The initial NES1 cDNA insert in the pSHlox-1 plasmid was sequenced from both ends using oligonucleotide primers corresponding to flanking SP6 and T7 promoters. Comparison of this sequence (418 bp) with published sequences (GenBank) revealed no matches, indicating that *NES1* represented a novel gene. To obtain full-length cDNA clones, the initial 418-bp NES1 cDNA was used as a probe to isolate additional cDNA clones from the 76NS λSHlox cDNA library. In addition, a second 76N cDNA library containing random- and oligo(dT)-primed cDNAs in the pGAD10 vector was screened with a NES1 oligonucleotide primer (nucleotides 674–657). The ends of the cDNA clones isolated by these two

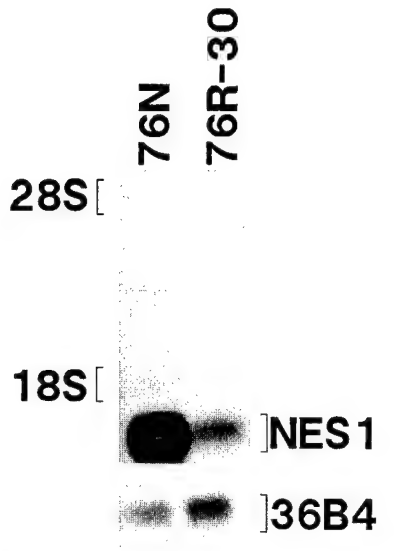

Fig. 1. Northern blot analysis of NES1 mRNA expression in 76N *versus* 76R-30 cells. Poly(A)$^+$ RNA from both cell lines was resolved on a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled, 418-bp NES1 probe and visualized by autoradiography. Locations of the rRNAs (28S, 4850 bp; and 18S, 1740 bp) are indicated. 36B4 was used as a loading control.

approaches were sequenced using primers corresponding to flanking vector sequences. Further sequences were determined using oligonucleotide primers corresponding to determined NES1 sequences (see "Materials and Methods" for primers). Using this strategy, a sequence of 1454 nucleotides was determined from overlapping cDNA clones in both directions (Fig. 2).

Translation of the determined nucleotide sequence of the NES1 cDNA clones revealed a 276-amino acid open reading frame starting with the first methionine codon at nucleotides 82–84, which occurs in a context (GCCATGA) that is compatible with a consensus Kozak sequence (A/GCCATGG; Ref. 23). The use of a less favorable ATG (nucleotide 154–156) would result in a 252-amino acid polypeptide. Based on the results of *in vitro* translation (see below), we favor the possibility that nucleotides 82–84 serve as the initiation codon (Fig. 2). Although the exact transcription start site of *NES1* has not been determined, the close correspondence of the determined sequence (1454 nucleotides) with the NES1 mRNA size (1.4 kb) suggests that the sequences upstream of the first methionine represent the 5′ untranslated region of the mRNA. This suggestion is reinforced by the comparison of NES1 to other homologous proteins (see below). Nucleotides 1417–1422 (ACTAAA) closely resemble a consensus polyadenylation signal (AATAAA; Ref. 24) and are followed by a stretch of 14 adenines after a space of 17 nucleotides. No other potential polyadenylation signals were discernible in the 3′ untranslated region, suggesting that nucleotides 1417–1422 represent the polyadenylation signal (Fig. 2).

Although the NES1 sequence is unique, data base comparison with available sequences revealed it to be highly homologous to a number of serine proteases. When the entire predicted NES1 polypeptide was used for homology comparisons (Lipman-Pearson protein alignment method), it showed 34–42% identity and 50–63% overall similarity with members of three distinct families of serine proteases: the trypsin family, the kallikrein family, and serine proteases that activate the kringle domain-containing growth factors (Fig. 3 and Table 1). Notably, the sequence identity was highest around the residues that are known to be involved in substrate binding and specificity and catalytic activity of the serine proteases (Figs. 3 and 4). In particular, all of the three residues (serine 231, aspartic acid 137, and histidine 86) that form the catalytic triad of serine proteases were found in relative locations similar to those of other serine protease (Fig. 4).

Sequence alignment also revealed a potential cleavage site (Arginine 42) at a site homologous to other serine proteases (Lysine or Arginine in most cases), suggesting that NES1 may be synthesized as a precursor (25). Additional similarities with serine proteases included a similar overall amino acid. length and 40–50% nucleotide sequence homology within the 5′ and 3′ untranslated regions. Altogether, these features strongly suggest that the *NES1* gene encodes a novel serine protease.

Interestingly, the kallikrein family serine proteases that showed the sequence homology to NES1 included prostate-specific antigen, the NGF 7S component, the NGF-γ component, tissue plasminogen activator, and EGF-binding protein (8–12, 26). Detailed analysis of NES1 did not reveal it to be closer to either the trypsin-like or kallikrein-like proteases, the members of which showed between 53 and 78% identity among themselves. The level of homology between trypsin and kallikrein family was similar to their homology with NES1 (30–40% identity). Furthermore, several amino acid stretches in NES1 showed poor homology to other proteases. These include amino acids 44–48, 122–130, and 148–151, and other regions throughout the predicted polypeptide sequence. In addition, the putative signal peptide sequences (first 14 or 15 amino acids after methionine) were not closely related to the corresponding sequence in trypsin, EGF-binding protein, or human tissue plasminogen activator (Fig. 3). Interestingly, both the NES1 and kallikrein family proteins (such as EGF-binding protein) showed a small insert (NES1, amino acids 126–136) immediately before His86 of the catalytic triad; however, this region and the immediate NH_2-terminal sequences of NES1 were not particularly homologous to either the kallikrein or trypsin family proteases. Taken together, these features suggest that NES1 is a distinct serine protease.

Hydropathicity analysis (Fig. 5) revealed the NH_2-terminal region to be quite hydrophobic, consistent with the possibility that this region may harbor a signal sequence analogous to other serine proteases (Fig. 3). Although the likelihood of this possibility is supported by the secretion of the NES1 protein (see below), this region did not show a strong homology to signal sequences of other serine proteases. Several evenly distributed hydrophobic regions throughout the NES1 polypeptide are consistent with a globular protein similar to other serine proteases.

***In Vitro* Translation.** The longest open reading frame of the NES1 cDNA (nucleotides 82–912) predicted a polypeptide of 30.14 kDa, whereas the use of the second methionine (nucleotide 154–156) predicted a polypeptide of 27.6 kDa. To experimentally determine the size of the polypeptide encoded by the NES1 cDNA clone, nucleotides 1–1069, which contained the predicted coding region and both potential initiation codons, were cloned in the pBluescript II KS vector in both sense and antisense orientations. The plasmid DNA was used for *in vitro* transcription, followed by translation in the presence of [^{35}S]methionine. As shown in Fig. 6, a 30-kDa polypeptide closely corresponding to the size predicted by the use of first methionine was observed in the translation reaction of the sense but not the antisense NES1 cDNA.

Tissue-specific Expression of NES1 mRNA. To assess the tissue distribution of NES1 expression, Poly(A)$^+$ RNA samples from various human tissues were hybridized with a NES1 probe corresponding to nucleotides 1–1069 of the full-length NES1 cDNA. As shown in Fig. 7, differential expression of the *NES1* gene was observed in the tissues examined. Relatively abundant levels of the 1.4-kb mRNA transcript were observed in the prostate, testis, ovary, small intestine, colon, and lung, with highest levels in the ovary. The pancreas showed abundant expression of a shorter (1.1-kb) mRNA transcript. In com-

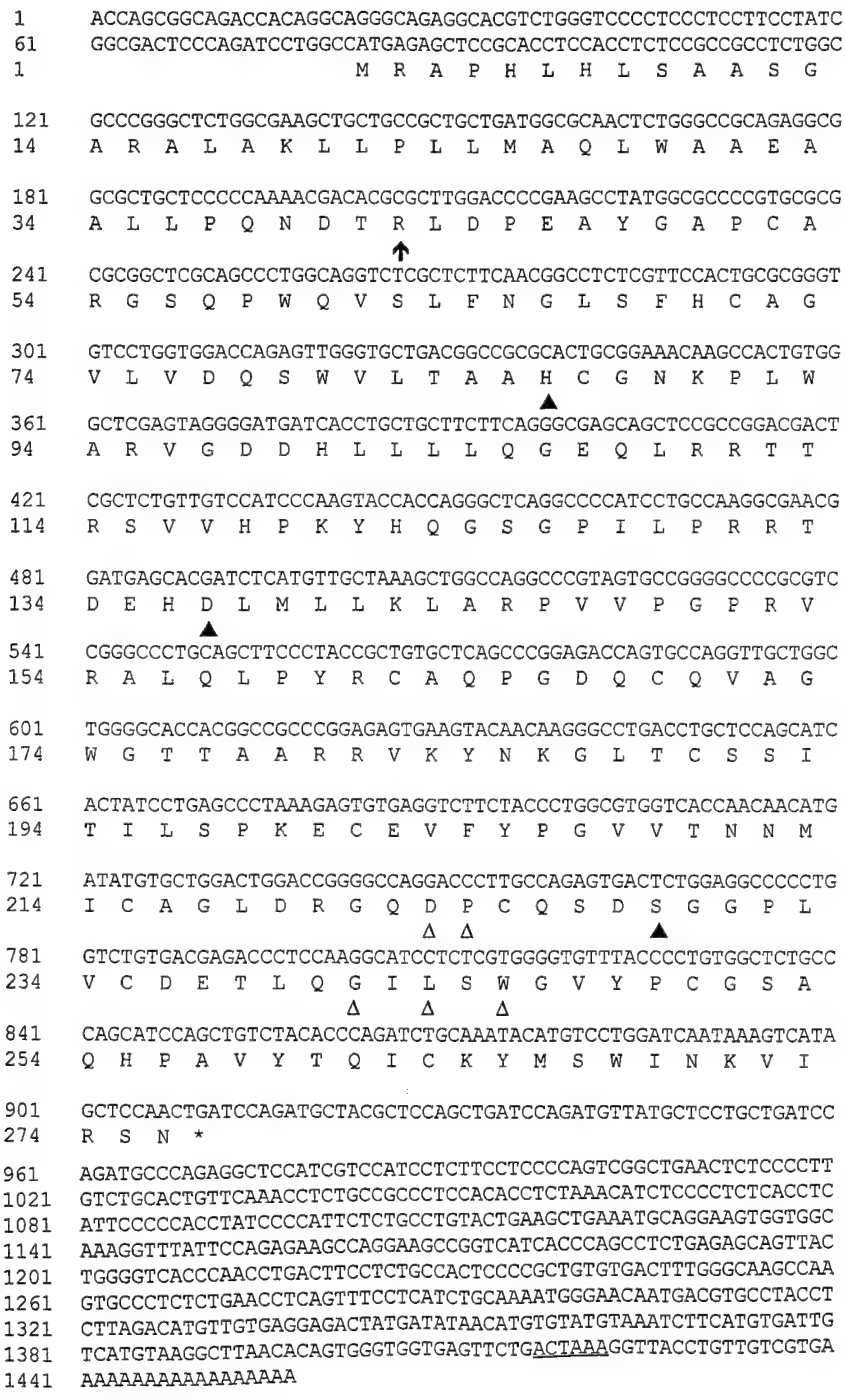

Fig. 2. Nucleotide sequence of the full-length *NES1* cDNA and the predicted amino acid sequence. Nucleotide sequence was obtained from multiple cDNA clones in both directions and is presented as a composite. ↑, cleavage site; ▲, catalytic triad; △, important amino acid in substrate binding; *, C terminus. Poly(A)$^+$ signal is *underlined*.

parison, NES1 expression in the thymus and heart was barely detectable and was essentially undetectable in peripheral blood leukocytes, brain, placenta, lung, liver, skeletal muscle, and kidney. Hybridization with the control probe 36B4 (27) demonstrated equal loading of mRNA in all lanes. These analyses show that NES1 mRNA is expressed in a large subset of human tissues.

Expression of *NES1* in Normal, Immortalized, and Tumor Mammary Cells. As described above, NES1 cDNA was isolated by virtue of its drastically reduced mRNA expression in MECs oncogenically transformed by γ-irradiation. To further assess the relationship between NES1 mRNA expression and mammary tumor progression, we analyzed a number of normal MECs, mammary fibroblasts, immortalized MECs, and established mammary tumor cell lines by Northern blot analysis. All of the normal (4 of 4) mammary epithelial cells expressed abundant levels of 1.4-kb NES1 mRNA, whereas none of the fibroblast cell strains (5 of 5) expressed detectable mRNA levels (hence, the designation normal epithelial cell specific 1 or NES1; Fig. 8 and data not shown). All of the MECs that have been induced to undergo preneoplastic transformation by HPV-16 E6 or E7 (10 of 10) expressed NES1 mRNA levels similar to those of normal MECs (Fig. 8 and data not shown). In contrast, a drastic decrease or complete loss of NES1 mRNA was observed in most (16 of 20) breast cancer cell lines examined (Fig. 8 and data not shown). Notably, all of the four breast cancer cell lines (21PT, 21NT, 21MT-1, and 21MT-2) that showed substantial NES1 mRNA expression (data not shown) are derived from a single breast cancer patient and are clonally related

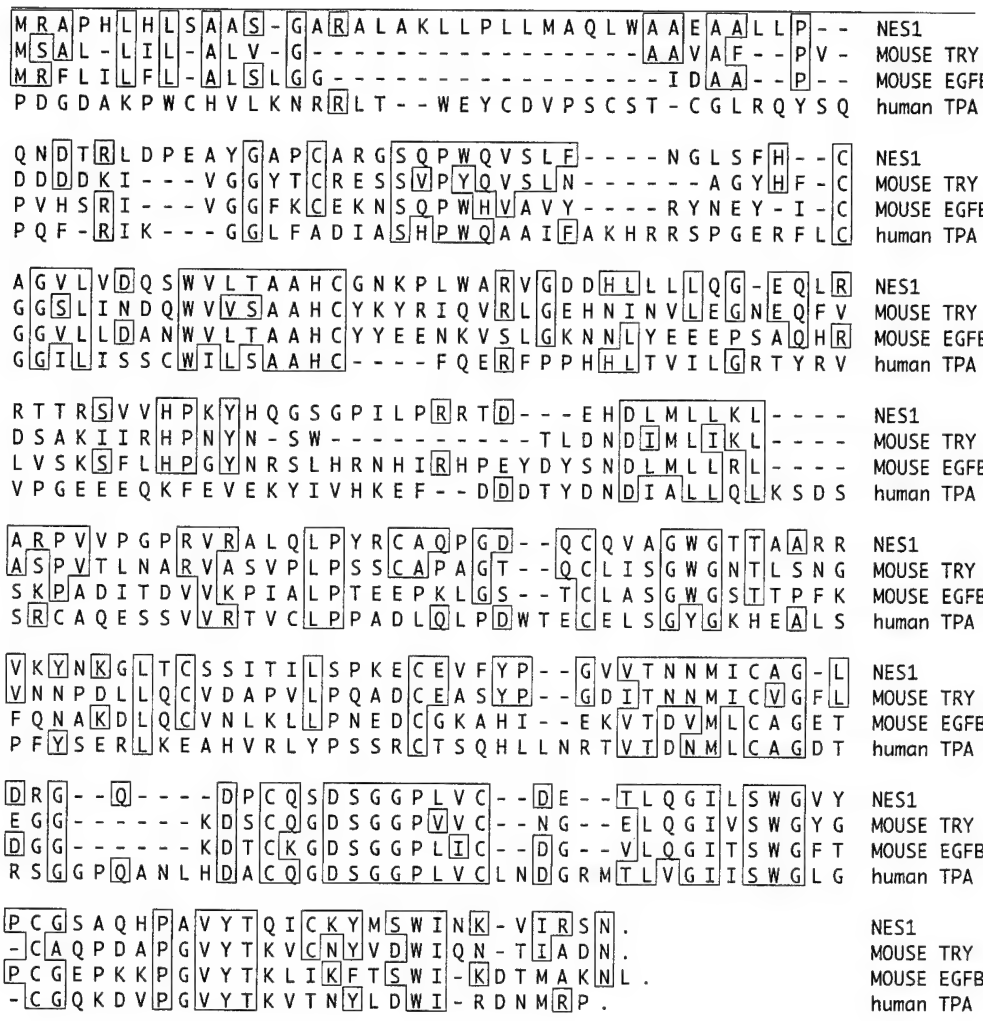

Fig. 3. Homology of the predicted NES1 amino acid sequences with serine proteases. Homology comparisons were made using the Clustal algorithm with a gap penalty of 3. Comparisons include NES1 amino acids 1–276, mouse preprotrypsin (*TRY*) amino acids 1–246, mouse EGF-binding protein (*EGFBP*) amino acids 1–261, and human tissue plasminogen activator (*TPA*) amino acids 270–562. ·, C terminus of the protein. Sequences in *boxes*, identity with the NES1 predicted amino acid sequence.

Table 1 *Amino acid homology of NES1 to other serine proteases*

Serine proteases	Similarity (%)	Identity (%)
Trypsin-like family		
Mouse preprotrypsin	63	42
Human pancreatic trypsinogen III	59	37
Human sapiens trypsinogen IV b	58	36
Kallikrein family		
Mouse nerve growth factor 7Sα	57	36
Mouse epidermal growth factor-binding protein	57	36
Mouse nerve growth factor γ	56	34
Human glandular kallikrein	54	38
Human kallikrein	53	35
Human prostrate specific antigen precursor	50	35
Kringle family		
Human tissue plasminogen activator	57	36
Human hepatocyte growth factor activator	53	34

(15). Reprobing of the blot with control probe 36B4 (27) showed that mRNA was indeed loaded in the lanes that showed little or no hybridization with the NES1 probe (Fig. 8). These results show that although NES1 mRNA expression is unaffected by immortalization (preneoplastic transformation) of mammary cells, it is drastically down-regulated in a large subset of established breast cancer cell lines.

Southern Blot Analysis of *NES1* Gene. To determine whether the decrease or loss of NES1 mRNA expression in oncogenically transformed cells was due to deletion or rearrangement of the gene, we performed Southern blot analyses of *Ban*II-, *Bgl*II-, or *Eco*RI-digested genomic DNA isolated from 76N, 76R-30, MCF-7, and MDA-MB-231 cells. As shown in Fig. 9, all four cell lines showed identical hybridizing bands of equal intensity. Therefore, loss of NES1 mRNA expression does not appear to be due to any major deletion or rearrangement of the gene. However, this analysis does not exclude the presence of more subtle mutations that may lead to loss of expression.

NES1 Is a Secreted Protein. Most of the proteins that showed homology to the predicted NES1 polypeptide contain a signal peptide at the NH_2 terminus (first 14–15 amino acids after methionine) and are secreted. Although the NH_2 terminus of NES1 did not show significant homology to the corresponding region of the other serine proteases, this region was strongly hydrophobic (Fig. 5), consistent with its being a signal peptide and suggesting that NES1 is a secreted protein. To examine this possibility, we generated a rabbit antiserum against a NES1-specific peptide (amino acids 120–137). This antiserum was used for Western blot analysis of cell lysates and culture supernatants derived from NES1 mRNA-positive and -negative MECs. As seen in Fig. 10, anti-NES1 antibodies specifically detected a 30-kDa polypeptide almost exclusively in the culture supernatants of all of the NES1 mRNA-positive cell lines. The 30-kDa polypeptide was not detected in the NES1 mRNA-negative cell lines. These data strongly support the possibility that NES1 is a secreted protein similar to other homologous serine proteases.

	86	137	223	224	227	228	229	230	231	241	243	245
NES1	H	D	D	P	S	D	S	G	G	G	L	W
Human Pancreatic Trypsinogen III	H ▲	D ▲	D △	S △	R	D	S ▲	G	G	G △	V △	W △

Fig. 4. Comparisons of putative active site and substrate-binding amino acids of NES1 with corresponding known residues in human trypsinogen III. ▲, catalytic triad and residues; △, residues important for substrate binding and specificity.

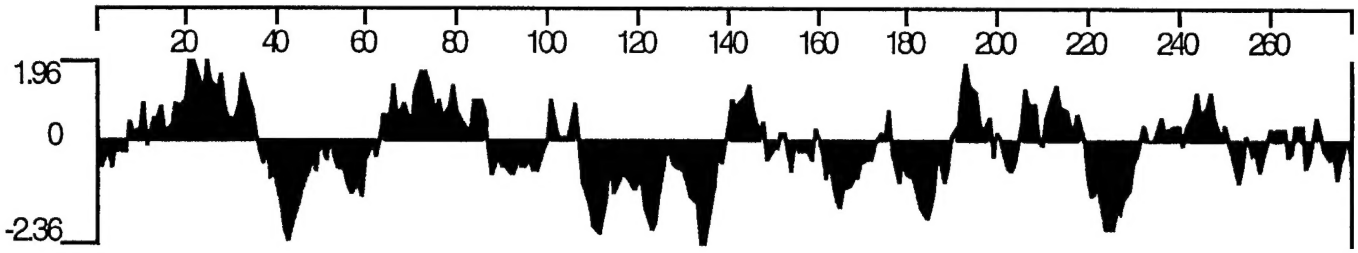

Fig. 5. Hydropathicity plot of the predicted NES1 polypeptide. Hydropathicity was calculated using the Kyte-Doolittle method in the DNASTAR program. Values greater than 0 represent hydrophobic regions, and those less than 0 represent hydrophilic regions.

DISCUSSION

Tumorigenesis represents a complex process in which altered expression of cellular genes plays an important role. Identification of these cellular genes is likely to enhance our understanding of the tumorigenic process and to provide diagnostic and/or therapeutic avenues in the future. Recently, we isolated a radiation-transformed tumorigenic derivative (76R-30) from normal human MEC strain 76N (6). This pair of cell lines provided a unique system to identify genes with altered expression during mammary tumorigenesis.

The present studies describe the isolation of a novel epithelial cell-specific cDNA, NES1, using subtractive hybridization between 76N and 76R-30 cells. As expected from its isolation from a subtracted cDNA library, the NES1 mRNA was abundantly expressed in 76N cells but was drastically reduced in 76R-30 cells. These analyses revealed a major mRNA species of 1.4 kb in 76N cells. Sequence analysis of the full-length cDNA clones showed that *NES1* is a novel gene. Data base comparison revealed that both the nucleotide and predicted amino acid sequences of *NES1* showed a strong homology (34–42% amino acid identity) to several distinct families of serine proteases, comparable to the level of homology between other protease families. In addition to sequence homology, all of the critical structural features of serine proteases were conserved in *NES1*. These include the invariant residues (histidine 86, aspartic acid 137, and serine 229) that form the catalytic triad of serine proteases (see Fig. 4 and Ref. 28). Additional residues surrounding serine 229, which are critical for substrate binding and specificity of serine proteases, are also conserved (Fig. 4). Compared with a 34–42% overall identity with known serine proteases, the sequences immediately around the critical catalytic center residues showed up to 95% identity with certain proteases. Finally, *NES1* also shows a potential tryptic cleavage site (Arginine 42) in a location similar to that seen in trypsinogen, at which it allows autocatalytic cleavage into active trypsin (25). Altogether, these conserved structural features strongly suggest that NES1 is a protease; however, this activity needs to be experimentally demonstrated.

Although the critical protease domains are conserved in NES1, it does show several unique structural features. Most notably, the NH_2 terminus of NES1 (which includes the putative signal peptide) and amino acids 44–48, 122–130, and 148–151 represent unique inserts not observed in other serine proteases. In addition, the level of homology between NES1 and other serine proteases (34–42% identity) is no more than the level of homology between other serine proteases belonging to distinct families, such as trypsin-like *versus* kallikrein-like proteins. In contrast, members of a single family typically showed 53–78% sequence identity. Thus, the divergence between NES1 and other serine proteases may have occurred early during evolution. This high level of divergence is consistent with unique biological functions of NES1.

Although the established physiological roles of the trypsin family of serine proteases are primarily in general protein catabolism (7), the functions of other serine proteases that share homology with NES1 are of special interest. For example, the NES1-related members of the glandular kallikrein family included NGF-γ and EGF-binding protein.

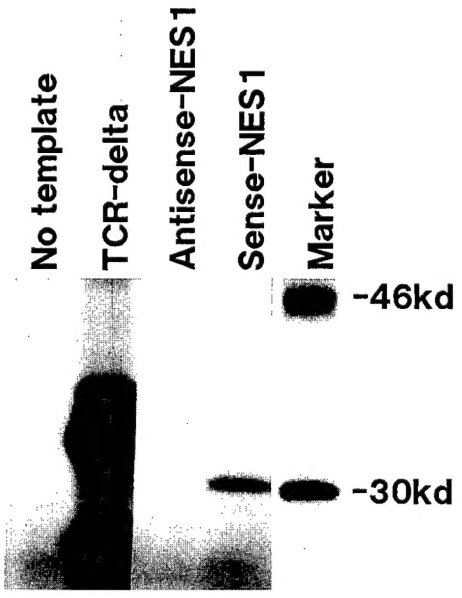

Fig. 6. *In vitro* translation of NES1 cDNA. NES1 cDNA fragment (1–1069 nucleotides) was cloned in pBluescript II KS vector in sense and antisense orientations, and plasmid DNA was used for *in vitro* translation using a coupled transcription-translation system in the presence of [^{35}S]methionine-cysteine (see "Materials and Methods"). ^{35}S-labeled, *in vitro*-translated products were run on a 10% SDS-PAGE. Note that sense but not antisense cDNA encoded a polypeptide of the predicted size (about 30 kDa). Human TCR-δ (expected size, 34 kDa; Ref. 47) was used as a positive control.

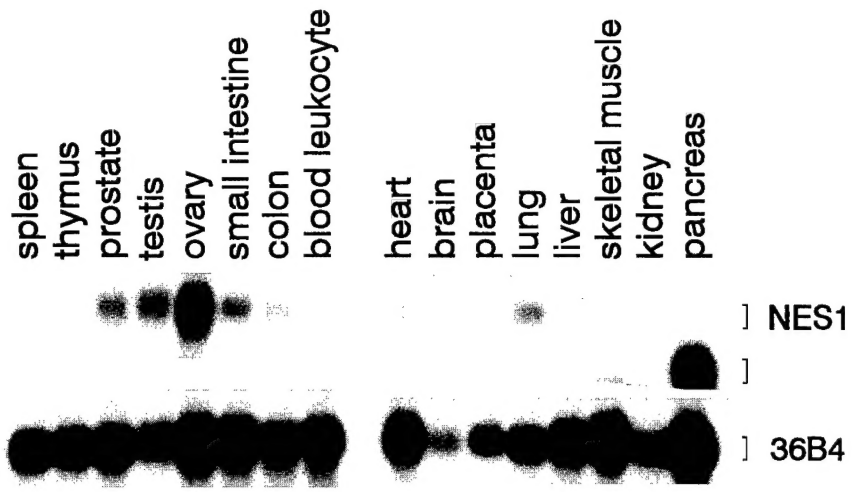

Fig. 7. Northern blot analysis of NES1 mRNA expression in various human tissues. Nylon membranes with 2 μg poly(A)$^+$ RNAs from each of the indicated human tissues were obtained from Clontech, hybridized with a ^{32}P-labeled NES1 probe, and visualized by autoradiography. 36B4 probe was used as a loading control.

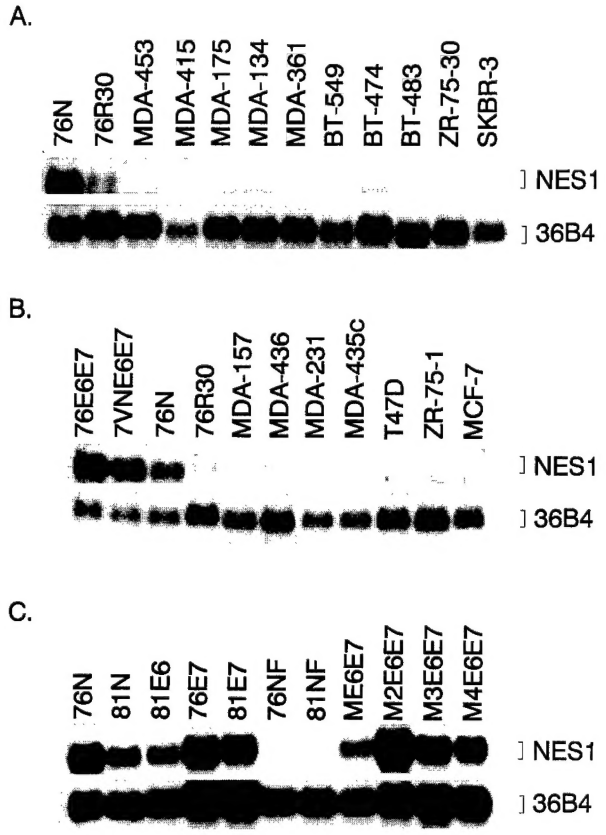

Fig. 8. Northern blot analysis of NES1 mRNA expression in cultured cell lines. Analysis was performed as described in Fig. 1 legend. Note a drastic decrease in NES1 mRNA levels in 76R-30 cells and almost complete loss in all mammary tumor cell lines. 36B4 was used as a loading control. *A–C* are separate blots. *A, 76N,* normal MEC; *76R-30,* radiation-transformed 76N MEC; remaining cell lines represent breast cancer-derived cell lines. *B, 76E6E7,* 76N cells immortalized by HPV-16 E6 and E7; *7VNE6E7,* 7VN cells immortalized by HPV-16 E6 and E7; *76N* and *76R-30,* as in *A;* remaining cell lines represent breast cancer-derived cell lines. *C, 76N* and *81N,* two normal mammary epithelial cell strains; *81E6,* HPV-16 E6-immortalized 81N cell; *76E7* and *81E7,* HPV-16 E7-immortalized 76N and 81N cells, respectively; *76NF* and *81NF,* two mammary fibroblast cell strains from two individuals designated 76 and 81, respectively; other cell lines are HPV-16 E6- and E7-immortalized human milk-derived epithelial cell lines from three separate specimens.

These proteins are known to proteolytically activate NGF-β and EGF, respectively (10, 29). NES1 also exhibited a similar level of homology to human prostate-specific glandular kallikrein, prostate-specific antigen and the pancreatic and renal kallikrein (26, 30, 31). All of these proteins are known to have protease activities against specific substrates, resulting in the liberation of a bioactive peptide or polypeptides (26, 30, 31). Finally, NES1 showed homology to human tissue plasminogen activator and the hepatocyte growth factor activator precursor (11, 12). These proteins are known to activate plasminogen and hepatocyte growth factor, respectively (11, 12). The latter proteins are members of the kringle protein family, characterized by the presence of a kringle domain (7). Significantly, hepatocyte growth factor binds to and activates a transmembrane receptor tyrosine kinase that regulates mitogenesis and differentiation of the target cells (32). These established activities of NES1 homologues raise the possibility that NES1 may serve to positively or negatively regulate cell growth or differentiation by cleavage of cell growth regulatory proteins.

NES1 cDNA was isolated by virtue of its decreased expression in a tumorigenic derivative (76R-30) of a normal MEC (76N). Therefore, it was of interest to determine whether established mammary cancer cells showed a down-regulation of its mRNA expression. Indeed, most established breast cancer cell lines showed drastically

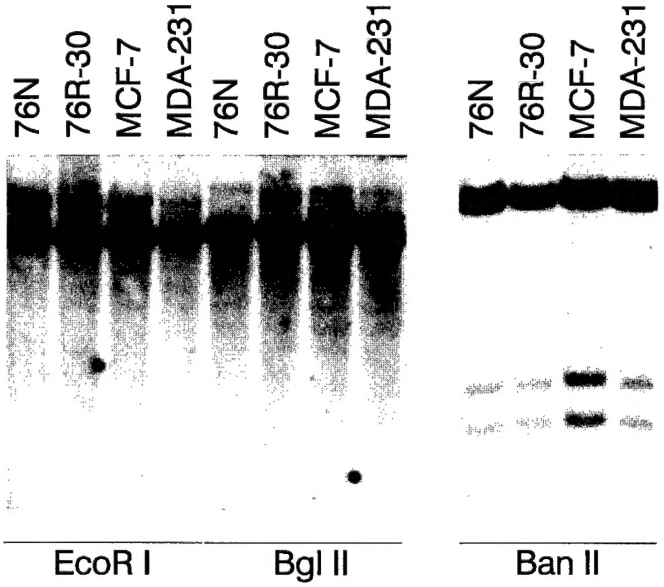

Fig. 9. Southern blot analysis of *NES1* gene. Ten μg genomic DNA from the indicated mammary cell lines were digested with *Eco*RI, *Bgl*II, or *Ban*II restriction enzymes, resolved on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled NES1 cDNA probe corresponding to nucleotides 1–1069. All cell lines show a similar pattern of bands of almost equal intensity.

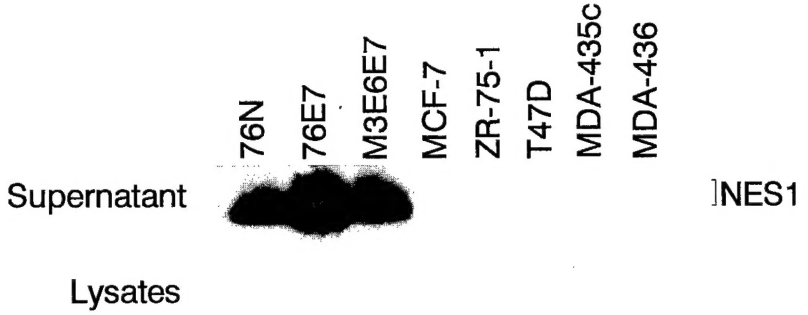

Fig. 10. Western blot analysis of NES1 protein. Aliquots of concentrated culture supernatant or cell lysates derived from number of indicated cell lines were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were immunoblotted with anti-NES1 antiserum followed by goat antirabbit IgG conjugated to horseradish peroxidase. Detection was by enhanced chemiluminescence.

decreased or absent NES1 expression. Interestingly, the levels or activities of several proteases show increases during malignant progression (33). In breast cancer cells, the increased levels of cathepsin D, plasminogen activator, and stromolysin (matrix metalloprotease III) have been shown to correlate with tumorigenic progression (34–36). A positive association with tumor aggressiveness has also been noted for a number of proteases, such as urokinase (37, 38), and protease inhibitors block tumor cell invasion (39). Contrary to these examples, however, NES1 expression negatively correlated with tumorigenic progression, suggesting that the products of NES1 proteolytic activity are likely to be involved in negative regulation of growth and/or tumorigenic behavior of mammary epithelial cells. Such a negative growth regulatory role of proteases is not unprecedented. For example, the cysteine protease interleukin 1β-converting enzyme, a homologue of the *Caenorhabditis elegans* death gene *ced-3*, is known to participate in apoptosis of cells (40–43). Interestingly, inhibitors of interleukin 1β-converting enzyme prevented the apoptosis of mammary epithelial cells that was induced by lack of extracellular matrix-derived signals (41). Recent studies using subtractive hybridization and differential display methods have identified other genes, such as a serpin homologue designated maspin and protease inhibitor elafin, the mRNA expression of which were decreased in tumor cells compared with normal mammary epithelial cells (44, 45). These genes have been designated class II tumor suppressor genes, to distinguish them from classical tumor suppressors, which are either mutated or deleted at the DNA level (46). Based on the loss of NES1 expression at the RNA level, this gene may also qualify for categorization as a class II tumor suppressor gene. However, this protein does not have any structural or predicted functional homology to other proteins, such as maspin or elafin, that have been grouped in this category.

Although NES1 has thus far been characterized primarily in the context of mammary epithelial cells, it is expressed in a number of other tissues, although not in all organs, suggesting a potentially organ-specific biological role for this protein. Interestingly, a smaller transcript was observed in the pancreas. The basis of this phenomenon remains to be elucidated. Furthermore, the mRNA expression was observed in epithelial cells but not in fibroblasts, indicating that in the context of a single organ, the function of NES1 may be restricted to certain cell types. However, definitive identification and characterization of NES1 targets will be required to fully unravel the mode of NES1 function. The *in vitro* studies of NES1 expression included mammary epithelial cells belonging to the "luminal" type (milk cells) as well as mammoplasty-derived normal epithelial cells, which may include both luminal and myoepithelial cells (14, 48, 49). However, direct studies of mammary tissues by *in situ* hybridization and immunohistochemistry will be required to directly ascertain the relative expression of NES1 in different mammary epithelial cell types.

We have cloned a novel putative serine protease gene, *NES1*, the expression of which is regulated during tumor progression of mammary epithelial cells. Based on the loss of its expression in breast cancer cells and its sequence homology to known serine proteases, we suggest that *NES1* may regulate the activity of growth stimulatory or inhibitory factors in MECs. Further studies of the protein encoded by this gene are likely to provide insights into mammary cell growth control and tumorigenesis.

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